AMPHETAMINES: NEW RADIOIMMUNOASSAY

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Received 3 August 1973

1. Introduction

Gas chromatography and thin-layer chromatography are the two main techniques that are now used for the detection of amphetamines in blood and urine [1, 2]. Since these two methods require solvent extraction and evaporation, they are relatively time-consuming and have a lack of sensitivity. For clinical and forensic purposes, a more rapid, specific, sensitive, and reliable technique for the determination of amphetamines in biological fluids is obviously needed. We now report the development of a radioimmunoassay for measuring nanogram levels of amphetamines in urine.

2. Materials and methods

N-(4-aminobutyl)methamphetamine was prepared by refluxing methamphetamine, containing a small amount of radioactive tracer, with one equivalent of *N*-(4-bromobutyl) phthalimide in dry absolute ethanol for 12 hr (fig. 1). One equivalent of 85% hydrazine hydrate aqueous solution was then added to the mixture and heated for another 2 hr. The reaction mixture was acidified, then filtrated and the filtrate extracted three times with chloroform. After readjusting the pH to 10 with sodium hydroxide, the free amine was separated from the aqueous layer.

The *N*-(4-aminobutyl) methamphetamine was coupled to bovine serum albumin (BSA) by a carbodiimide method (fig. 1). To the mixture of 1.36 g BSA and 1.34 g *N*-(4-aminobutyl)methamphetamine in 50 ml water at pH 6, we added 7.66 g of 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) in 12 ml of water. The mixture was stirred for 3 hr at room temp. The reaction mixture was then dialyzed against distilled water for 4 days. Based upon the

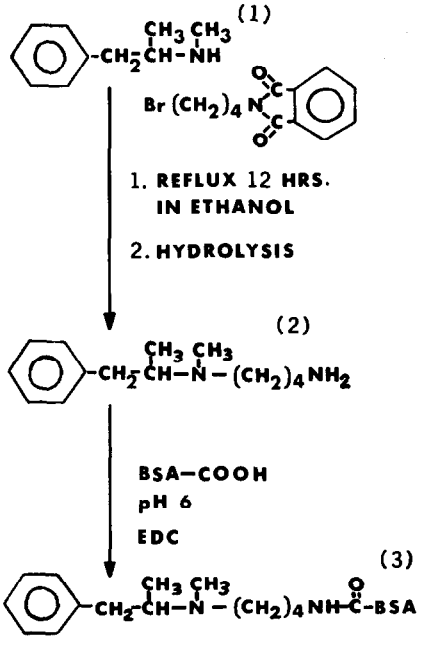

Fig. 1. Preparation of *N*-(4-aminobutyl) methamphetamine and conjugation to BSA. Structure (1) is methamphetamine; structure (2) is *N*-(4-aminobutyl)methamphetamine; structure (3) is *N*-(4-aminobutyl) methamphetamine–BSA conjugate. EDC: 1-Ethyl-3-3(3-dimethylaminopropyl)-carbodiimide.

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amount of the radioactive tracer bound to the protein carrier, the degree of conjugation was calculated to be 93 mol of methamphetamine/mol of BSA carrier (mol.wt. = 68 000).

Rabbits were inoculated subcutaneously with 1 mg of *N*-(4-aminobutyl) methamphetamine–BSA conjugate emulsified with 50% Freund's complete adjuvant in saline for 8–10 weeks. The hapten-binding capacity of the antiserum was determined by a saturated ammonium sulfate method. Serial dilutions of antiserums were incubated with 0.25 pmole (approx. 2200 cpm) of [^{3}H] amphetamine (New England Nuclear, 6.24 Ci/mmole as the sulfate) at 4°C overnight. An equal volume of a saturated ammonium sulfate solution was added to all tubes after incubation. After centrifugation, the supernatant containing free amphetamine was dissolved in 10 ml of scintillation fluid and the radioactivity was counted in a Nuclear Chicago spectrometer. The standard curves were fitted to the equation:

$$(C_h + C_h^*)b = -(1/K)\cdot(b/f) + C_a \qquad (1)$$

a modification of Scatchard equation [3], to obtain the affinity constant K and the concentration of antibody binding sites C_a. In eq. 1 C_h^* and C_h are respectively the concentrations of labeled and unlabeled amphetamine; while b and f stand for the fractions of bound and free amphetamine, respectively. We obtained $K = 2 \times 10^7$ to 2×10^9 M^{-1} and $C_a = 2 \times 10^{-8}$ to 6×10^{-8} M for our methamphetamine antiserums

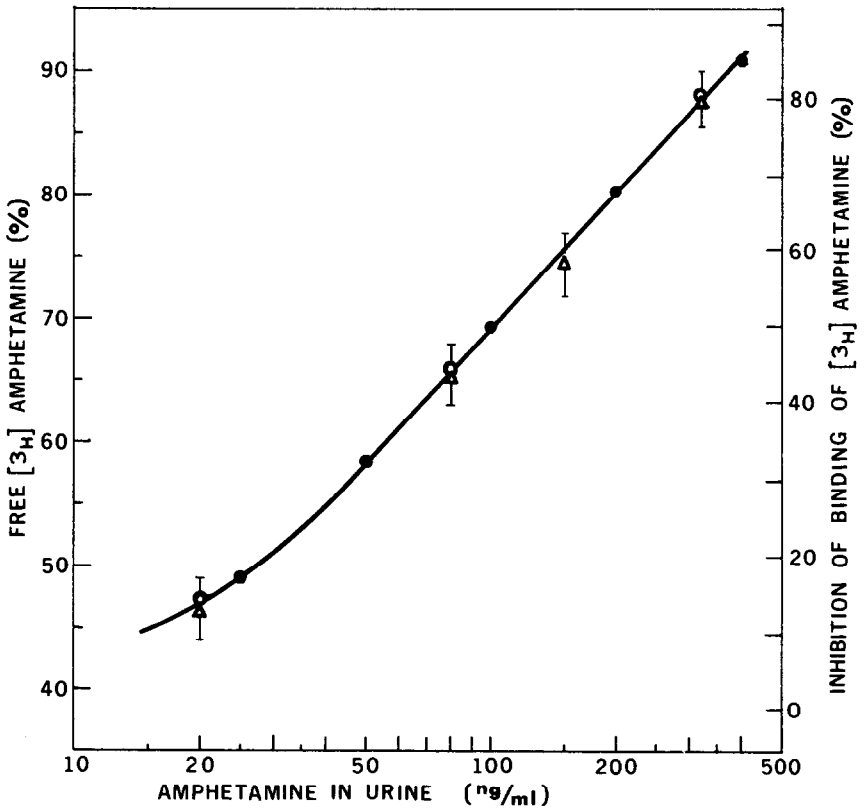

Fig. 2. Displacement of binding of [^{3}H] amphetamine by nonradioactive amphetamine. The affinity constant and the concentration of binding sites were calculated from the black dotted (•) points to be 2.4×10^7 M^{-1} and 6.2×10^{-8} M, respectively. Using these two constants and the eq. 1 the line was generated. The different mark points were run at different times. The vertical bar indicates the standard deviation (± S.D.) of six to eight runs. In each assay, 0.050 ml urine was incubated with 0.200 ml of antiserum and fixed amounts of [^{3}H] amphetamine in 0.01 M phosphate-buffered solution.

at 1:250 dilution. Good fitting of the standard curves to the equation [1] indicates that the binding of amphetmaine to the antibody obeys the law of mass action and that all antibody-binding sites in the antiserum may be considered as equivalent.

For assays of amphetamine in urine, 0.050 ml of urine, containing various amounts of unlabeled amphetamine, was incubated with 0.200 ml of antiserum and a fixed amount (2200 cpm of [^{3}H]amphetamine (labeled in 0.01 M phosphate-buffered solution (pH 7.6). After separation of the free- and bound-labeled amphetamine by the saturated ammonium sulfate method, the radioactivity of free labeled amphetamine was counted.

3. Results and discussion

The principle of the radioimmunoassay is based upon the displacement of bound labeled amphetamine in the antiserum by unlabeled amphetamine. A tube that contained labeled amphetamine and antiserum, but no unlabeled amphetamine, measured minimum radioactivity. The addition of samples with increasing amounts of unlabeled amphetamine to fixed amounts of labeled amphetamine and antiserum resulted in increasing displacement of [^{3}H]amphetamine from binding to the antibody. Thus we observed an increase in the percentage free [^{3}H]amphetamine and the radioactivity (figs. 2 and 3). The basic principle is similar to that of the radioimmunoassays for morphine [4] and barbiturates [5]. We found that the length of incubation could be reduced from overnight to 30 min without sacrificing the sensitivity as shown in fig. 3. Although in the latter case the amount of displacement was lower and the control of timing was essential for good reproducibility, it substantially saved the overall turn-around time for the assay. In all cases, the method detected amphetamine in urine at 30 ng/ml with better than 99% confidence. This corresponds to 1.5 ng of amphetamine in 50 μl of urine, the actual amounts used in the tests.

The antibody bound equally well to amphetamine and methamphetamine, the two main central nerve stimulants. This was predicted since these two drugs are different only in a methyl substituent on the amino group to which the bridge chain was linked. Additional substituent on the α-carbon and the β-

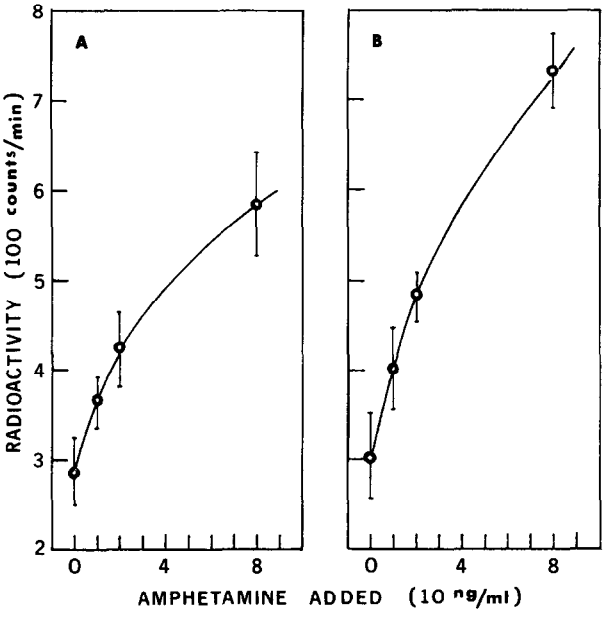

Fig. 3. Displacement of binding [^{3}H]amphetamine by non-radioactive amphetamine with two incubation times: A) Incubated for 30 min at room temp; B) Incubated overnight at 4°C. The vertical bar on each point represents the three standard deviations (± 3 S.D.) of eight runs.

carbon, however, reduced the cross reactivity* to 0.25 and 0.15, respectively, as observed for mephenterine and norephedrine. The cross reactivities for α-methylbenzylamine, tyramine, and isoproterenol were further down to 0.0006, 0.0008 and 0, respectively. The data indicate that the phenyl group is the major moiety which determines the antibody specificity. We also compared these results with those of antiserums produced from the conjugate prepared by direct coupling of amphetamine with BSA without extending the bridge chain (fig. 1). The extra bridge chain actually enhanced both the affinity and specificity of the antibody. This similar effect seems to explain the high affinity and specificity of the phenobarbiturate-antibody reported earlier [5].

* Cross reactivity: 1/(the amounts of substance which displaced [^{3}H]amphetamine equivalent to that displaced by 1 ng of unlabeled amphetamine) using 1:250 dilution of antiserum.

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